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ORIGINAL ARTICLE

Prevention of chronic allograft nephropathy with vitamin D*

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Summary

Chronic allograft nephropathy (CAN) is the leading cause of late allograft loss in kidney transplantation. Interstitial fibrosis and glomerulosclerosis are characteristic of CAN. Transforming growth factor beta-1 (TGFβ-1) is associated with both of these histologic findings in the transplant setting. Recent studies have suggested that vitamin D signaling pathways may interact with and regulate TGFβ-1 mediated events. We examined the efficacy of 1,25-dihydroxyvitamin D_3, the active metabolite of vitamin D [1,25-$(OH)_2D_3$], the active metabolite of vitamin D, as monotherapy to prolong allograft survival and preserve renal function in a rat model of CAN, the Fisher 344 to Lewis model. Recipients went without treatment or were treated with cyclosporine A (CSA; 10 days) or 1,25$(OH)_2D_3$ (1000, 500 or 250 ng/kg/day). Grafts were harvested at the time of rejection or at 24 weeks post-transplant. A portion of the graft was processed for histology and immunohistochemistry and a second portion was analyzed for protein expression by western blotting. Not only did 1,25-$(OH)_2D_3$ treatment significantly prolong graft survival, but it also prevented histological changes associated with CAN. 1,25-$(OH)_2D_3$ treatment significantly decreased Smad 2 expression. This TGFβ signaling molecule is likely involved in fibrosis. Moreover, 1,25-$(OH)_2D_3$ treatment increased Smad 7 expression, an important feedback molecule in the TGFβ-1 signaling pathway. This suggests that 1,25-$(OH)_2D_3$ interacts with TGFβ-1 in limiting histological injury in this model of CAN. Furthermore, 1,25-$(OH)_2D_3$, treatment increased expression of matrix metalloproteinase 2 (MMP-2), thus directly affecting levels of another important matrix molecule. Taken together our data suggests that 1,25-$(OH)_2D_3$ mitigates CAN in this model by altering TGFβ-1 and matrix-regulating molecules.

Introduction

Chronic allograft nephropathy (CAN) is characterized by fibrotic changes throughout the allograft including tubulointerstitial fibrosis, glomerulosclerosis, tubular atrophy, and concentric neointimal hyperplasia. CAN appears to be an irreversible process. It leads to graft failure and ultimately results in re-transplantation or dialysis. The mechanisms underlying the development of CAN are unknown, but likely involve a complex interaction between humoral and cellular immune responses, ischemia/perfusion injury and cytokine expression. Transforming growth factor beta-1 (TGFβ-1), in particular, plays a prominent role in CAN. The involvement of this cytokine in CAN has been reviewed recently [1]. Several transplant studies have demonstrated correlations between TGFβ-1 expression and the development of interstitial fibrosis and glomerulosclerosis in kidney transplant recipients [2–5].

1,25-dihydroxyvitamin D_3, [1,25-$(OH)_2D_3$] is the active metabolite of vitamin D. Interestingly, it regulates immune responses and prevents the development of a number of autoimmune diseases in rodent models [6].

1,25-$(OH)_2D_3$ also prevents acute allograft rejection [7]. Many of these effects may result from 1,25-$(OH)_2D_3$'s inhibition of dendritic cell maturation and its effects on regulatory T cell development [8,9] or T helper phenotype [10]. Thus, 1,25-$(OH)_2D_3$ may be a useful immunosuppressive agent for transplantation.

It is important to recognize 1,25-$(OH)_2D_3$'s mechanism of action in attempting to understand its effects in a transplant setting. 1,25-$(OH)_2D_3$ traverses the cytoplasmic membrane where it binds the vitamin D receptor (VDR). VDR or VDR complexed with the retinioic acid receptor [retinoid X receptor (RXR)] then travels to the nucleus where it functions in conjunction with other co-activator/repressors as a transcription factor to differentially affect the expression of various genes, depending on cellular phenotype, cell cycle, and cellular activation [11]. There is a direct link between the 1,25-$(OH)_2D_3$ and TGFβ-1 signaling pathways. TGFβ-1 binding to its cell surface receptor results in the phosphorylation of the receptor-activated TGFβ signaling proteins (Smads) 2/3 that then interact with the co-Smad, Smad 4, to form a heterodimeric complex that translocates to the nucleus to regulate gene expression. We and others have shown that Smad 3 and Smad 7, each form a complex with the VDR, *in vivo* and *in vitro* [12,13]. Such a complex in the nucleus could act as transcriptional regulator that can bind the promoters of new target genes or it could alter the affinity of the transcriptional regulatory complex for the promoters of traditional target genes. This suggests that 1,25-$(OH)_2D_3$ could regulate TGFβ-1-mediated gene and protein expression and, therefore alter TGFβ-1 effect's in CAN.

Here we examined the effects of 1,25-$(OH)_2D_3$ therapy in the Fisher to Lewis renal allograft model, a model of CAN. Our results suggest that 1,25-$(OH)_2D_3$ is effective in prolonging allograft survival and limiting CAN in this model in association with discrete changes in TGFβ-1 signaling molecules.

Materials and methods

Animals

Donor and recipient rats (>250 g) were obtained from Harlan Sprague Dawley, Indianapolis, IN, USA. Recipient animals were placed on experimental diet containing 0.47% Ca [14] 7 days prior to transplantation. Recipients were divided into groups that received experimental diet alone or experimental diet containing 1,25-$(OH)_2D_3$ (250, 500, or 1000 ng/rat/day) or cyclosporine A (CSA) 1.5 or 5 mg/kg/day i.p. for 10 days. Animals were maintained on diet until the time of rejection or graft harvest at 24 weeks and were housed singly to ensure equal food intake. All care and use of laboratory animals followed the National Institutes of Health (NIH) (NIH publication No. 86-23) guidelines. 1,25-$(OH)_2D_3$ was prepared, dissolved in ethanol and placed in the experimental diet as previously described [7]. Ethanol was also added to the diet prepared for non-1,25$(OH)_2D_3$ treated recipients. 1,25-$(OH)_2D_3$ was the generous gift of Dr Hector DeLuca, Department of Biochemistry, University of Wisconsin, WI, USA. CSA (Sandimmune i.v.;1.5 or 5 mg/kg/day for 10 days.) was diluted in saline and given i.p.

Transplantation

The Fisher to Lewis model of CAN has been described [15]. Briefly, donor kidneys obtained from male Fisher 344 rats were flushed with 10 ml cold University of Wisconsin preservation solution and stored at 4 °C while the recipient was prepared. Total cold ischemic time did not exceed 30 min. Lewis male recipients were transplanted with either a Fisher 344 or Lewis kidney following left native nephrectomy. The donor renal artery, vein and ureter were anastomosed to the recipient renal artery, vein and ureter. The right native kidney was removed 10 days post-transplant. Graft function was monitored by serum creatinine and urinary protein determinations. Serum creatinine determinations were performed by the University of Wisconsin Hospital and Clinics Clinical Laboratories. Grafts were harvested at the time of sacrifice because of declining renal function or at 24 weeks.

Urinary protein

Proteinuria was assessed every 2 weeks. Animals were placed in metabolic cages for 24 h and urine collected. Protein excretion was determined using a dye-binding assay (quanTtest red, Quantimetrix Corp., Redondo Beach, CA, USA) according to manufacturer's instructions with minor modifications. Briefly, 20 μl of the urine sample was diluted with 1X phosphate-buffered saline (PBS) in a twofold series dilution in 96-well flat-bottom microtiter plates (Corning, NY, USA). The final dilution was 1:32. 125 μl quanTtest red reagent was added and the protein concentration in the samples measured by reading the absorbance at 600 nm and compared with the absorbance of a 50–0.062 mg/ml rat albumin/globulin protein standard on a V_{max} Kinetic microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA). Data was analyzed with Softmax Pro-software (Molecular Devices Corp).

Western blot analysis

A portion of the snap frozen graft containing both cortex and medulla was ground with a mortar and pestle. For each 100 mg of tissue, the sample was resuspended in 400 μl of lysis buffer (10 mM Tris base, 150 mM NaCl, pH 8.0)

containing protease inhibitors (P2714 1:1000, Sigma Chemical Co., St Louis, MO, USA) and homogenized (PowerGen 125, 1 min) (Fisher Scientific, Pittsburgh, PA, USA). Triton X100 was then added to 1% and the sample place on ice, for 30 min. The solublized sample was then spun at 4 °C for 15 min at 15 000 *g*. The supernatant was collected and stored at −80 °C. Sample protein concentrations were determined using the Micro BCA assay (Pierce Chemical Co., Rockford, IL, USA) according to manufacturer's instructions, except that the assay was performed in half area plates combining 35 μl sample with 70 μl of reagent. Samples (30 μg/well) were resolved on a 10% reducing acrylamide gel and transferred to a nitrocellulose membrane using a semi-dry transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked overnight at 4 °C in Blotto B [1% dry milk, 1% bovine serum albumin (BSA), 0.05% tween 20 in PBS] plus 0.05% sodium azide. Membranes were washed with PBS tween (6X, 10 min) followed by addition of diluted primary antibody [Blotto B, 5% dry milk, 1 h at RT with rocking; rabbit anti-Smad 2, 3, 6 (Zymed Corp., San Francisco, CA, USA; 1:6000); goat anti-Smad 7 (Santa Curz, Santa Cruz, CA, USA; 1:2500) or mouse anti-tubulin clone Ab-4 (Neomarkers, Fremont, CA, USA (1:14 000)]. Membranes were again washed and diluted secondary HRP-conjugated antibody added [anti-rabbit IgG-HRP 1:32 000 (Transduction Labs, Lexington, KY, USA); anti-goat IgG-HRP 1:2500 (Transduction Labs); anti-mouse IgG-HRP 1:240 000 (Transductions Labs)]. The membranes were washed and (four 10-min washes with PBS-tween followed by two 10-min PBS washes) and then developed using the SuperSignal West Pico Chemiluminescent substrate according to manufacture's instruction (Pierce Chemical Co.) Fold changes were expressed as the ratio of Smad protein levels to tubulin level relative to the untreated allogeneic control. A negative value was assigned if the ratio of the group to the allogeneic control was <1.

Enzyme linked immunoabsorbant assay

The TGFβ-1 and MMP 2 levels were quantified by antigen-capture enzyme linked immunoabsorbant assay (ELISA) from the same lysates used in the Western blots. A flat bottomed, half area EIA/RIA A/2 plate (Costar, Cambridge, MA, USA) was coated overnight at 4 °C with 25 μl monoclonal primary antibody [anti-TGFβ-1, IgG, 1:1000 dilution in carbonate buffer, pH 9.7, TGFβ-1 E_{max} (ImmunoAssay; Promega Inc., Madison, WI, USA); anti-MMP 2, 2.5 μg/ml (clone 1A10; R&D Systems, Minneapolis, MN, USA)]. After blocking with 1X block buffer at 37 °C for 35 min, 25 μl of cell lysate (diluted 1:10 in the lysis buffer) was added to the wells. A standard curve was generated by performing twofold serial dilutions of standard active TGFβ-1 antigen (diluted 15.6–1000 pg/ml, Promega Inc.) or MMP 2 (0.5–100 ng/ml, R&D Systems) in lysis buffer. The plate was incubated for 2 h at RT with shaking and then washed extensively with wash buffer (0.05% Tween 20 in PBS) followed by PBS. The TGFβ-1 ELISA was then developed according to the manufacturer's instruction. For the MMP 2 ELISA, biotinylated detection antibody, clone 101721 (25 μl well at 1.4 μg/ml, R&D Systems) was added and incubated at RT (1 h, with shaking). The antibody was biotinylated using a Mini-Biotin-XX Protein Labeling Kit (F-6347) according to manufacturer's instruction (Molecular Probes, Eugene, OR, USA). Following further washing, an avidin-peroxidase conjugate was added (25 μl/well at 1:5000) for 30 min at RT. The color reaction was developed by the addition of 25 μl of the 3, 3′, 5, 5′-tetramethylbenzidine (TMB)/hydrogen peroxidase substrate solution (KPL, Gaithersberg, MD, USA). Color development was stopped after approximately 10 min by the addition of 25 μl TMB stop solution. Absorbance was measured at 450 nm on a V_{max} Kinetic microplate reader (Molecular Devices). To measure total TGFβ-1 in the sample, acid activation was performed: 1 μl of 1N HCl was added to the harvested supernatant sample (diluted 1:5 in PBS) and incubated at RT for 15 min. One microlitre of 1 M NaOH was then added to neutralize the acid. Acid activated samples were then assayed by antigen-capture ELISA after a further 1:10 dilution in sample buffer.

Immunohistochemistry and histological scoring

Formalin fixed, paraffin embedded rat kidneys were sectioned to 4 μm. Sections were re-warmed on day of staining at 60 °C for 10 min, then deparaffinized in xylene for 30 min followed by rehydration. Sections were washed twice in distilled water and antigen retrieval performed. Briefly, sections were soaked in 100 mM citrate buffer, pH = 6.0 for 10 min at 90 °C and then heated to 115 °C for 20 min. Sections were allowed to cool and then washed in PBS and blocked for 10 min with Biocare Super Sniper9 (BS966L) followed by diluted primary antibody [TGFβ-1 (Promega Corp) G1221, 1:150; Smad 2, rabbit anti-Smad 2 (Zymed) 1:200; anti-MMP 2 (R&D Systems) 1:200] at 4 °C overnight. Slides were washed (3 × 5 min, PBS) and secondary antibody applied (1 h at RT, MachII Biocare goat anti-rabbit with polymer spacer; RHRP52OH, Walnut Creek, CA, USA). Slides were developed with Pierce's Stable Peroxide buffer (catalogue no. 1855910) and Pierce's Stable Metal enhanced DAB solution (catalogue no. 1856090). Grafts were scored on a scale of not detectable (ND) to +++ (intense staining).

For histological scoring, H&E sections were prepared from paraffin embedded grafts. Grafts were scored for

interstitial fibrosis, glomerulosclerosis, tubular atrophy and intimal hyperplasia (IH) using a scale of 0–3 (0 = no pathology; 1 = <25% involvement, mild; 2 = 25–50% involvement, moderate: and 3 = >50% involvement, severe).

Statistical methods

Differences in graft survival were tested with a log-rank test. A one-way ANOVA was used to test differences between groups in interstitial fibrosis, glomerulosclerosis, tubular atrophy and IH ratings. If the overall *F*-test was significant, least squares mean values and standard errors were obtained for each treatment group. All pairwise comparisons between groups were calculated; this corresponds to Fisher's protected least significant difference test. The ANOVA was run with procedure GLM in SAS 6.12 for Windows (SAS Institute, Cary, NC, USA). Differences in protein expression were compared by *t*-test and one-way ANOVA. Differences in serum creatinine and urinary protein were compared using the Wilcoxon Rank Sum test.

Results

1,25-$(OH)_2D_3$ prolongs allograft survival and decreases the severity of CAN

Dietary 1,25-$(OH)_2D_3$ (1000 ng/rat/day, monotherapy, $n = 9$) significantly prolonged graft survival in allogeneic recipients (Fig. 1a; $P = 0.0031$) in comparison with allogeneic untreated controls ($n = 14$). When 1,25-$(OH)_2D_3$ was reduced to 500 ng/rat/day significant prolongation of graft survival was sustained ($P = .0.0009$, $n = 10$), but 250 ng/day did not have as pronounced effect on graft survival ($P = 0.4$, $n = 8$). Prolonged graft survival at 1000 or 500 ng/rat/day was not statistically different from recipients treated with CSA (1.5 or 5 mg/kg/day for 10 days) or syngeneic controls.

An early acute rejection episode typically occurs within 2 weeks post-transplant in this model. This is prevented with short-term CSA monotherapy (5 mg/kg for 10 days; Fig. 1b). Low-dose CSA (1.5 mg/kg/day for 10 days) limits this rejection episode. Treatment with 1,25-$(OH)_2D_3$ (1000 ng/day), in the absence of CSA, however, attenuated the early acute rejection episode with only a minimal increase in serum creatinine at 2 weeks post-transplant (Fig. 1b; $P = 0.0005$ versus untreated allogeneic control; $P = 0.02$ versus the low dose CSA allogeneic group). There was a possible dose-response effect although as the response to 250 ng/day of 1,25-$(OH)_2D_3$ was similar to untreated allogeneic controls. When grafts were harvested at 1 or 2 weeks post-transplant, histology revealed acute rejection in the untreated allografts which was absent in allografts treated with 1,25$(OH)_2D_3$ (1000 ng/rat/day, data not shown). There was no evidence of delayed graft function (data not shown). As shown in Table 1, most recipients gained an average of 25–30% of their weight

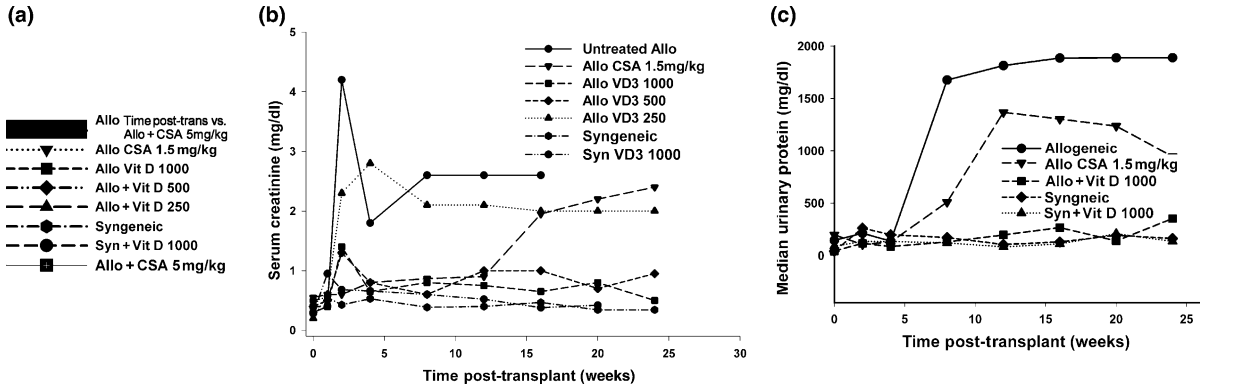

Figure 1 (a) Prolongation of renal allograft survival with 1,25-$(OH)_2D_3$. Lewis recipients were transplanted with either a Lewis or F344 renal graft. Recipients were either untreated (syngeneic, $n = 8$; allogeneic, $n = 10$) or received 250 ($n = 6$), 500 ($n = 14$), or 1000 ng/rat/day ($n = 9$) 1,25-$(OH)_2D_3$ in the diet beginning on day 7. CSA (5 mg/kg/d, $n = 9$ or 1.5 mg/kg/day, $n = 6$) was begun on the day of transplant and given for 10 days. Significant prolongation of graft survival was obtained with 1,25$(OH)_2D_3$ at 1000 ng/rat/day ($P = 0.0031$,) and 500 ng/rat/day ($P = 0.0009$) but not 250 ng/rat/day ($P = 0.4$) Graft function was monitored with serum creatinine and urinary protein. (b) Serum creatinine levels in transplant recipients. Serum creatinine levels in whole blood were determined at the times indicated. 1,25$(OH)_2D_3$ (1000 ng/rat/day) significantly reduced serum creatinine early post-transplant $P = 0.0005$ versus untreated allogeneic control; $P = 0.02$ (*) versus the low dose CSA allogeneic group. (c) Urinary protein secretion following transplantation. Recipients were placed in metabolic cages for 24 h, urine collected and protein concentration determined. 1,25$(OH)_2D_3$ at 1000 ng/rat/day significantly reduced urinary protein in comparison to untreated allogeneic recipients [$P = 0.003$, $P \leq 0.4$, $P = 0.01$, $P = 0.002$, and $P = 0.006$ at 8, 12, 16, 20 and 24 weeks post-transplant] or allogeneic recipients treated with low dose CSA (1.5 mg/kg/day; for 10 days: $P = 0.21$, $P = 0.13$, $P = 0.02$, $P = 0.002$ and $P = 0.01$ at 8, 12, 16, 20 and 24 weeks post-transplant, respectively). Most recipients gained an average of 25–30% of their weight at the time of transplant except in the untreated allograft group in which recipients gained only 5%.

Table 1. Weight gain in recipients at the time of graft harvest.

Group	At transplant	At graft harvest	Weight gain	*P*-value versus untreated allogeneic
Low dose CSA	372 ± 36	435 ± 30	63 ± 53	<0.15
Allo untreated	361 ± 24	371 ± 71	19 ± 42	
Syngeneic	382 ± 50	485 ± 5	116 ± 41	<0.001
Syngeneic 1000	355 ± 23	430 ± 31	75 ± 28	<0.02
Allogeneic 1000	274 ± 25	341 ± 43	63 ± 53	<0.016

from the time of transplant during the 6-month post-transplant course except animals in the untreated allograft group. Those recipients gained only 5% of their weight. At the time of the early rise in creatinine (during acute rejection) there was no difference in weight between the groups.

We also determined urinary protein excretion following transplantation. Monotherapy with 1,25-$(OH)_2D_3$ 1000 ng/rat/day ($P = 0.003$, $P <= 0.4$, $P = 0.01$, $P = 0.002$, and $P = .006$ at 8, 12, 16, 20 and 24 weeks post-transplant, respectively; Fig. 1c) or 500 ng/rat/day (data not shown) significantly lowered urinary protein excretion in comparison with untreated allogeneic controls or allogeneic recipients treated with low-dose CSA (1.5 mg/kg/day; for 10 days: $P = 0.21$, $P = 0.13$, $P = 0.02$, $P = 0.002$ and $P = 0.01$ at 8, 12, 16, 20 and 24 weeks post-transplant, respectively).

Histological examination of the untreated allografts (Fig. 2a) or allografts treated with low-dose CSA (Fig. 2b) (24 weeks) demonstrated interstitial fibrosis, glomerulosclerosis and neointimal hyperplasia, features characteristic of CAN. 1,25-$(OH)_2D_3$ treatment inhibited the development of these pathological features (Fig. 2c). Again, there appeared to be dose-response effect. Recipients treated with 250 ng 1,25$(OH)_2D_3$/rat/day manifested evidence of a mild to moderate cellular infiltration (Banff IA or IIA) in seven of eight allografts (Fig. 2d). However, there also was a narrow therapeutic window. Despite inhibition of rejection and stable function, it was apparent that some calcium deposits were present in recipients treated with 1000 ng of 1,25-$(OH)_2D_3$. While serum calcium levels remained elevated in the 500 ng/day group (12.2 ± 0.8 mg/dl vs. 11.1 ± 1.1 mg/dl low dose CSA allogeneic recipients at 24 weeks) histological examination showed reduced calcium deposition. Serum calcium levels were not significantly different ($P = 0.8$). Trichrome staining revealed decreased collagen deposition in 1,25-$(OH)_2D_3$-treated recipients and preservation of glomerular structure (Fig. 2e–h). Semi-quantitative histological analysis of the all grafts revealed a significant reduction in interstitial fibrosis and IH from allogeneic recipients treated with 1,25$(OH)_2D_3$ with 1000 or 500 ng/rat/day but not 250 ng/rat/day (Table 2). Tubular atrophy was significantly reduced in grafts from recipients treated with 500 ng/rat/day 1,25$(OH)_2D_3$. This was also observed in grafts from recipients treated with 1000 ng/rat/day, with the difference approaching statistical significance ($P = 0.093$). Tubular atrophy was not reduced in grafts from recipients treated with 250 ng/rat/day 1,25$(OH)_2D_3$.

1,25-$(OH)_2D_3$ treatment does not alter TGFβ-1 bioactive protein expression

An antigen-capture ELISA was performed using an antibody specific for bioactive TGFβ-1 to determine protein expression. Interestingly, bioactive protein (2.3 fold) expression was elevated in the high dose CSA-treated allogeneic recipients (5 mg/kg). There was no significant difference in the level of bioactive TGFβ-1 protein expressed between any of the groups in comparison with the untreated allogeneic controls (Fig. 3; $n = 5$). As shown in Fig. 4, immunohistochemistry for bioactive TGFβ-1 revealed staining in tubular areas of 1,25-$(OH)_2D_3$-treated and nontreated allogeneic grafts. However, little bioactive TGFβ-1 expression was observed in the glomeruli of 1,25-$(OH)_2D_3$-treated grafts in contrast to the untreated allogeneic control grafts. Semi-quantitiative analysis of immunostaining suggest a small increase in the intensity of bioactive TGFβ-1 staining in interstitial and tubular areas of untreated allogeneic grafts in comparison with grafts from recipients treated with either 500 or 1000 ng/rat/day 1,25$(OH)_2D_3$ (Table 3).

1,25-$(OH)_2D_3$ treatment altered allograft Smad expression

Immunoblotting revealed a marked decrease in Smad 2 (Fig. 5; $n = 3$ or more for each group, $P = 0.04$) protein levels in recipients treated with either 1000 (216-fold) or 500 ng/rat/day (208-fold, $P = 0.03$, $n = 3$ data not shown) in comparison with allogeneic untreated controls. In grafts from recipients treated with 250 ng/rat/day 1,25$(OH)_2D_3$ Smad 2 was reduced (7.2 fold) in comparison with untreated allogeneic controls, however the difference was not significant ($P = 0.11$). Smad 3 protein levels were similar in allogeneic recipients regardless of treatment but were decreased in comparison with syngeneic controls. Smad 7 protein levels were increased in

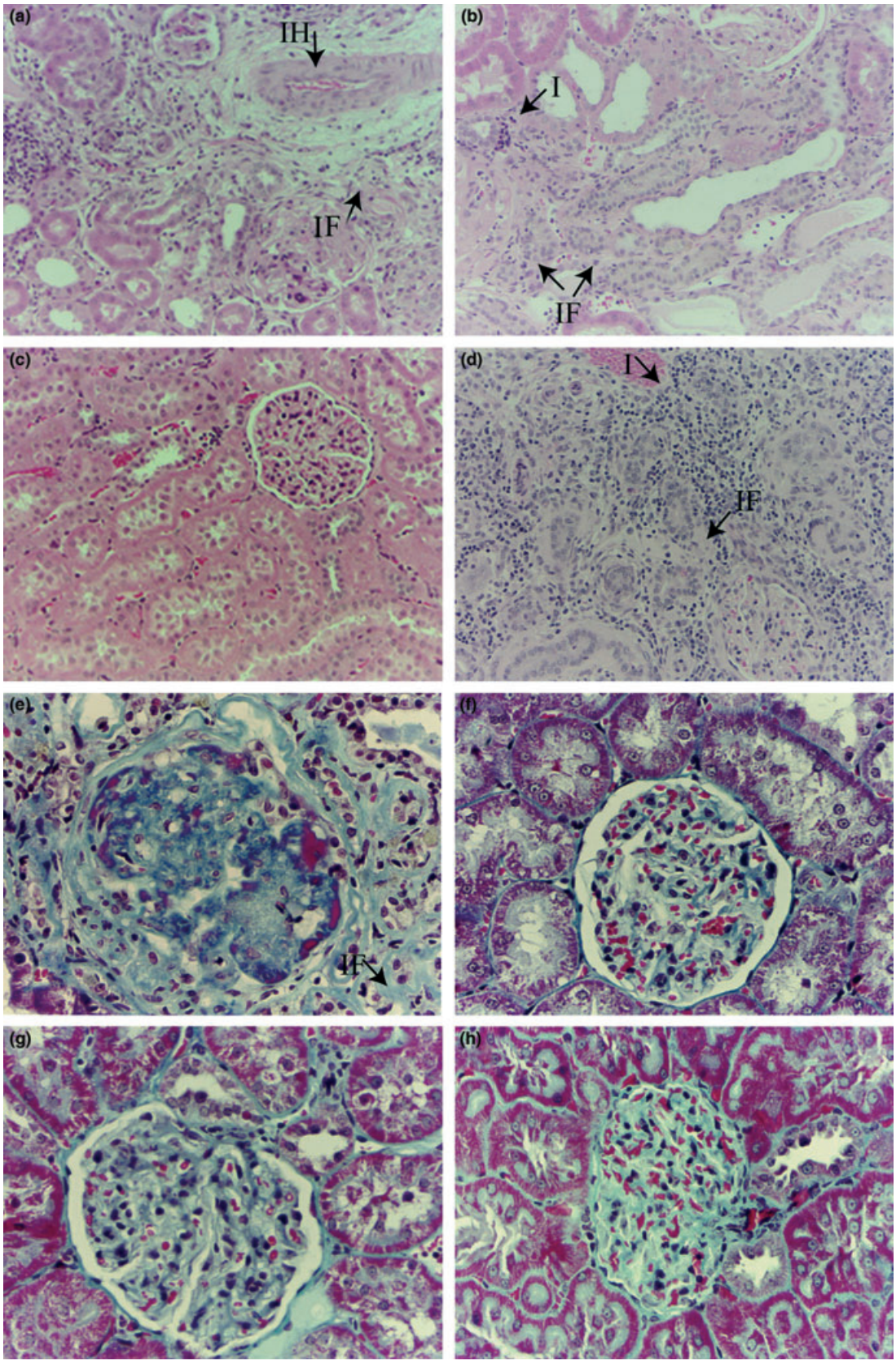
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Table 2. Histological score of grafts.

Group	Interstitial fibrosis	Glomerulosclerosis	Tubular atrophy	Intimal hyperplasia
Syngeneic 9 ($n = 8$)	0.4 ± 0.2 (0.0001)	0.1 ± 0.2 (0.0004)	0.5 ± 0.2 (0.0001)	0.4 ± 0.2 (0.0001)
Syngeneic 1000 ($n = 8$)	0.4 ± 0.3 (0.0001)	0.3 ± 0.3 (0.002)	0.7 ± 0.3 (0.001)	0.0 ± 0.2 (0.0001)
Syngeneic 500 ($n = 6$)	0.6 ± 0.3 (0.0012)	0.2 ± 0.3 (0.002)	0.8 ± 0.3 (0.005)	0.2 ± 0.4 (0.0001)
Syngeneic 250 ($n = 7$)	0.2 ± 0.3 (0.0001)	0.4 ± 0.3 (0.01)	0.8 ± 0.3 (0.005)	0.0 ± 0.2 (0.0001)
Allogeneic Untreated ($n = 10$)	2.9 ± 0.2 (0.013)	2.6 ± 0.2 (0.003)	2.9 ± 0.2 (0.012)	2.7 ± 0.2 (0.064)
Allogeneic CSA (1.5 mg/kg, $n = 7$)	2.0 ± 0.3	1.5 ± 0.2	2.0 ± 0.3	2.2 ± 0.2
Allogeneic 1000 ($n = 9$)	1.4 ± 0.2 (0.0923)	1.1 ± 0.2 (0.31)	1.6 ± 0.2 (0.31)	1.5 ± 0.2 (0.028)
Allogeneic 500 ($n = 14$)	1.3 ± 0.2 (0.043)	0.9 ± 0.2 (0.092)	1.2 ± 0.2 (0.025)	1.5 ± 0.2 (0.011)
Allogeneic 250 ($n = 8$)	2.0 ± 0.3 (1.0)	1.5 ± 0.3 (1.0)	2.2 ± 0.3 (0.671)	2.2 ± 0.2 (1.0)

P-value versus low dose allogeneic CSA (1.5 mg/kg) is shown in parenthesis. Grafts were scored on a scale of 0–3 (0, not present; 1, 25%, mild; 2, 20–50%, moderate; 3, >50%, severe).

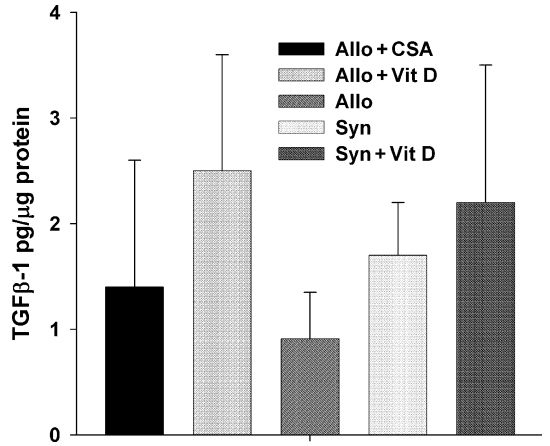

Figure 3 Bioactive TGFβ-1 protein levels in allografts are not changed by $1,25(OH)_2D_3$ treatment. Grafts were harvested at the time of rejection or at 24 weeks post-transplant. Bioactive TGFβ-1 levels were determined by a TGFβ-1-specific antigen-capture ELISA. There were no statistically significant differences between the various groups. Data are the mean ± SD ($n = 5$).

both 1,25-$(OH)_2D_3$-treated (4.3 fold, $P = 0.02$) and CSA-treated (5 mg/kg/day; 5.4-fold $P = 0.03$) allogeneic grafts in comparison with untreated allogeneic grafts (Fig. 5). However, Smad 7 in allogeneic recipients was decreased in comparison with syngeneic controls. Smad 6 protein expression was decreased in both 1,25-$(OH)_2D_3$ and CSA-treated allograft recipients and was unchanged in syngeneic recipients in comparison with allogeneic untreated control animals. Smad 7 protein expression was also elevated in the $1,25(OH)_2D_3$-treated syngeneic recipients. Immunohistochemistry confirmed the marked reduction of Smad 2 expression in $1,25(OH)_2D_3$-treated grafts (Fig. 4 and Table 3).

1,25-$(OH)_2D_3$ altered allograft MMP expression

Given the effects on TGFβ-1 signaling, we were also interested in whether 1,25-$(OH)_2D_3$ affected mechanisms of matrix deposition. Therefore, we analyzed MMP-2 protein expression. Total MMP 2 protein expression was increased in both syngeneic and allogeneic 1,25-$(OH)_2D_3$-treated groups and was unchanged in the CSA-treated allogeneic recipients in comparison with untreated allogeneic animals (Fig. 6; $n = 3$ for all groups). Immunohistochemistry for MMP 2 showed that the increased expression in $1,25(OH)_2D_3$-treated allografts occurred primarily in the tubular epithelium (Fig. 4).

Discussion

Our data clearly suggest that 1,25-$(OH)_2D_3$ therapy improved allograft function. In conjunction, the expression of molecules directly related to extracellular matrix

Figure 2 1,25-$(OH)_2D_3$ treatment prevents histopathological changes associated with CAN. (a) H&E from an untreated allograft. Note the cellular infiltrates (I), interstitial fibrosis (IF) and neointimal hyperplasia (IH) in a small artery. Magnification 200×. (b) H&E from an allograft that received low dose CSA (1.5 mg/kg/day for 10 days). (c) H&E from an allograft treated with 500 ng/rat/day 1,25-$(OH)_2D_3$. There is little or no interstitial fibrosis, with significantly decreased cellular infiltration. (d) H&E from allograft treated with 250 ng/rat/day $1,25(OH)_2D_3$, Banff score IIA. The graft contains numerous cellular infiltrates and interstitial fibrosis. Magnification 200× (a–d). Trichrome Mason stained section from an untreated allograft (e), a $1,25(OH)_2D_3$-treated (500 ng/rat/day) allograft recipient (f) an untreated syngeneic graft (g) and syngeneic graft treated with 1,25-$(OH)_2D_3$ (1000 ng/rat/day) (h). Note the lack of collagen deposition, interstial fibrosis, tubular atrophy and preservation of glomerular structure in the 1,25-$(OH)_2D_3$-treated allograft. Magnification 400× (e–h).

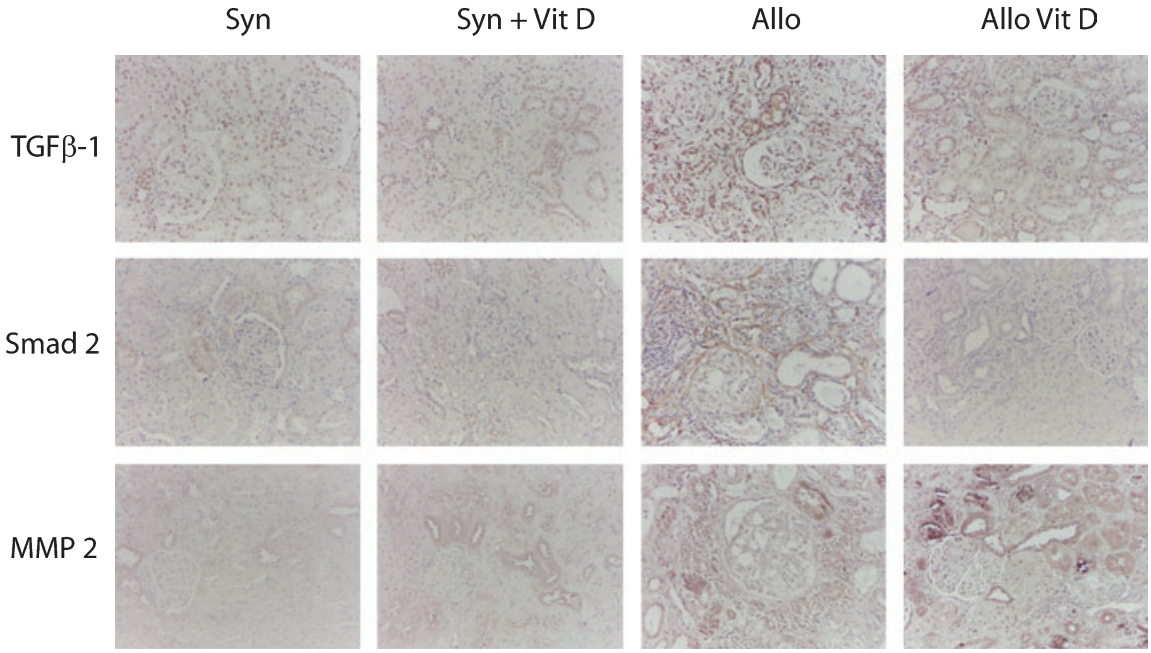

Figure 4 Immunohistochemical analysis of grafts for TGFβ-1, Smad 2 and MMP 2 protein expression. 1,25$(OH)_2D_3$ treatment decreased glomerular bioactive TGFβ-1 expression. Tubular staining was observed in all grafts. Smad 2 protein expression was dramatically reduce by 1,25$(OH)_2D_3$ treatment. Increased MMP 2 protein expression was observed primarily in the tubular epithelium. Magnification 200×.

Table 3. Immunohistochemical analysis of grafts.

	TGFβ-1			Smad 2			MMP 2		
Group	Tub	Glom	Inst	Tub	Glom	Inst	Tub	Glom	Inst
Syngeneic	±	+	+	+	+	+	+	ND	ND
Syngeneic 1000	+	+	+	ND	ND	ND	++	+	ND
Allogeneic	++	+	++	+++	ND	+++	++	ND	ND
Allogeneic 1000	+	+	+	+	ND	++	+++	+	ND

Grafts were scored on a scale of not detectable (ND) to +++ (intense staining).

(ECM) remodeling in this model of CAN were also modified.

Chronic allograft nephropathy is characterized by interstitial fibrosis, glomerulosclerosis, tubular atrophy, and concentric IH in arteries (vasculopathy) [16–18]. Many of these processes have been associated with the TGFβ-1 expression [1]. This cytokine is a potent stimulator of ECM deposition, stimulating in kidney tissue, collagen and fibronectin synthesis by many cell types [19–21]. Within the glomerulus, Nicholson *et al.* [22] have noted a specific elevation of TGFβ-1 expression following the development of CAN. They also noted a correlation with collagen type III deposition. We have observed that the beneficial effects of 1,25-$(OH)_2D_3$ treatment is in part the prevention of histological changes associated with CAN. Immunostaining of 1,25-$(OH)_2D_3$-treated allografts demonstrated reduced expression of TGFβ-1 specifically in the glomeruli although whole kidney TGFβ-1 levels were unchanged based on the ELISA results. Together with the observed decrease in collagen deposition in 1,25-$(OH)_2D_3$-treated allografts, these data suggest that 1,25-$(OH)_2D_3$ may modulate TGFβ-1-mediated fibrotic events.

There is an important interaction between the TGFβ-1 and the 1,25-$(OH)_2D_3$ signaling pathways [12,23,24]. Yanagisawa *et al.* [12] have demonstrated *in vitro* that Smad 3 functions as a co-activator to the VDR forming a heterodimeric complex with Smad 3/Smad 4 in cells over expressing VDR while Subramaniam *et al.* [24] have suggested that co-activation requires close proximity of the VDR and the Smad binding response elements. We have observed complex formation between the VDR and Smad 3 in renal graft cell lysates derived from 1,25-$(OH)_2D_3$-treated animals suggesting an *in vivo* interaction as well. In addition, we and others have shown that the VDR interacts with Smad 7 both *in vitro* [23] and *in vivo*. Following 1,25-$(OH)_2D_3$ treatment we observed a dramatic reduction in the Smad 2 expression with minimal change in Smad 3 levels in allograft recipients. The changes in Smad 2 protein expression that we observed are consistent with recent findings of Li *et al.* [25] describing TGFβ-1-mediated fibrotic changes in a renal tubular

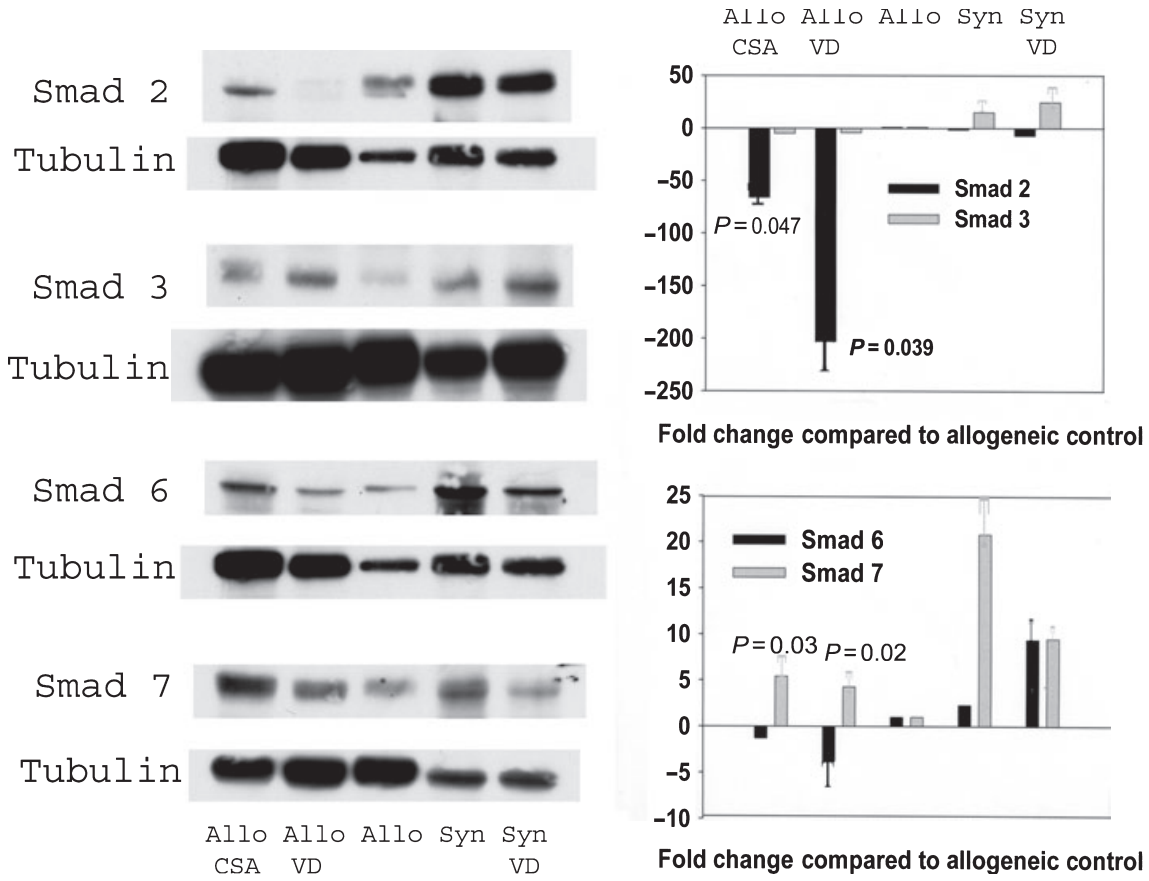

Figure 5 1,25-$(OH)_2D_3$ treatment significantly inhibits Smad 2 protein expression. Renal lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with specific anti-Smad antibodies and appropriated HRP-conjugated secondary antibodies. Signal was detected with chemiluminesent substrate. Following exposure, X-ray films were scanned and the amount of expression compared to that of tubulin as an internal control using Scion Image software. (a) A Western blot for smad 2, 3, 6 7 and tubulin. (b) Fold changes in protein expression. Fold changes are expressed as the ratio of Smad protein levels to tubulin level relative to the untreated allogeneic control. A negative value was assigned if the ratio of the group to the allogeneic control was less than one. 1,25$(OH)_2D_3$ significantly decreased Smad 2 protein expression ($P = 0.04$).

epithelial cell line where the effects of TGFβ-1 were mediated by Smad 2. One possible mechanism is that complex formation of VDR with Smad 2 may signal ubiquintination and degradation. In contrast, inhibitory Smad 7 expression was increased. This could result from a TGFβ-1 autocrine feedback loop or stabilization of Smad 7 expression as a VDR complex. Taken together, these data suggest that 1,25-$(OH)_2D_3$-mediated reduction in Smad 2 and increased Smad 7 protein expression may be an important mechanism in preventing CAN.

The matrix metalloproteinases (MMPs) family of proteins are responsible for the remodeling of ECM [26]. Their expression is regulated, in part, through the Smad family of transcription factors. In concert with the tissue inhibitor of matrix metalloproteinases (TIMPs), they regulate aspects of matrix deposition and uptake. We previously demonstrated changes in MMP expression in transplant patients with CAN [27]. A recent report characterizing the role of Smad 2 and Smad 3 in TGFβ-1 signaling in Smad 2- and Smad 3-deficient fibroblasts demonstrated the dependence of MMP 2 expression on Smad 2 [28]. In the studies reported here, we observed increased MMP 2 expression. In addition to altering the TGFβ-1 signaling pathway, one possible mechanism by which 1,25-$(OH)_2D_3$ mitigates CAN is by altering the MMP/TIMP balance through regulation of the Smads. Alternatively, VDR may act as transcription factor to directly regulate MMP/TIMP expression. Within the cell, free 1,25-$(OH)_2D_3$ traverses the cytoplasmic membrane where it binds the VDR. Binding of 1,25-$(OH)_2D_3$ to the VDR results in phosphorylation of the VDR and the ability to bind specific DNA sequences either as a homodimer or a heterodimer with the RXR [29]. VDR or VDR-RXR binding to response elements may differentially affect the

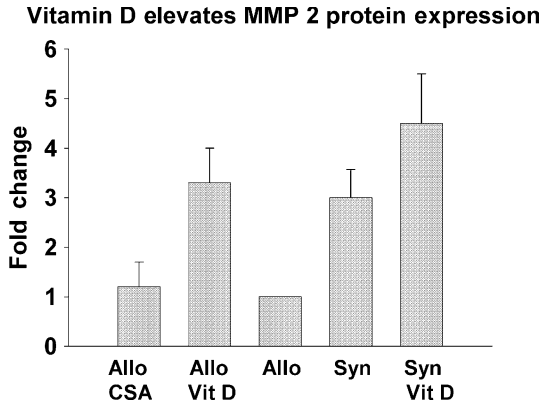

Figure 6 1,25-$(OH)_2D_3$ treatment alters MMP protein expression. Protein expression was quantified by Western blot analysis. Fold changes are expressed relative to the allogeneic untreated control as described above. 1,25$(OH)_2D_3$ treatment significantly increased MMP 2 protein expression in 1,25$(OH)_2D_3$-treated allogeneic recipients in comparison to untreated allogeneic controls ($P = 0.04$).

expression of various genes, depending on cellular phenotype, cell cycle, and cellular activation [11]. Thus, 1,25$(OH)_2D_3$ may directly regulate MMP expression. It is likely that the effect of 1,25$(OH)_2D_3$ on the histopathological changes of CAN is the result of the interaction of multiple mechanisms. The relatively small changes in Smad 7 and MMP 2 expression when combined with the dramatic reduction in Smad 2 expression could result in significant alterations in histopathology as we observed. As Smad 2 is required in the Smad 3/4 complex for translocation to the nucleus, the end result could be a dramatic reduction in the induction of genes required for matrix remodeling. We have also observed *in vitro* using MMP promoter reporter constructs in a renal proximal tubular cell line that 1,25$(OH)_2D_3$ directly regulates MMP 2 expression (D.A. Hullett, unpublished observations).

Progressive glomerular diseases including CAN, glomerulonephritis, diabetic nephropathy, and focal segmental glomerulosclerosis are often characterized by mesangial cell proliferation and the subsequent ECM accumulation [22,30]. Within the glomerulus, mesangial cells in these disease settings undergo a phenotypic change. They up-regulate smooth muscle α-actin expression and acquire fibroblast characteristics. They also secrete collagens normally absent in the matrix e.g. collagen I and IV, in addition to secreting increased amounts of collagen III. Abe *et al.* [31] have suggested that 1,25-$(OH)_2D_3$ or non-hypercalcemic analogs of 1,25-$(OH)_2D_3$ regulate mesangial SMC phenotypes. Additionally, data in a five of six nephrectomy model and an IgA glomerulonephritis model demonstrated that 1,25-$(OH)_2D_3$ treatment prevented the development of glomerulosclerosis [32,33]. Strikingly, we observed decreased glomerular collagen deposition and inhibition of proteinuria in 1,25-$(OH)_2D_3$-treated allograft recipients. In contrast to allogeneic control grafts, these grafts showed almost no glomerular bioactive TGFβ-1 expression by immunostaining. Studies by Li *et al.* [25] and Chen *et al.* [34] have suggested that TGFβ-1-mediated changes in mesangial cell phenotype and collagen synthesis can be blocked by increased Smad 7 expression. We observed increased Smad 7 expression in 1,25-$(OH)_2D_3$-treated recipients. Taken together these data suggest a potential pathway for preservation of glomerular structure and function by 1,25-$(OH)_2D_3$.

Most studies have demonstrated marginal prolongation of graft survival by 1,25-$(OH)_2D_3$ [35]. In all cases, significant hypercalcemia was observed. With one exception, 1,25-$(OH)_2D_3$ in these studies was administrated by daily or alternate day i.p. injection. Unfortunately, the efficacy of 1,25-$(OH)_2D_3$ is limited by its short half-life *in vivo* when delivered i.p. In contrast to these studies, we demonstrated increased allograft survival in both a murine nonvascularized and a rat vascularized heart allografts when 1,25-$(OH)_2D_3$ was delivered orally [7]. There is an important caution to consider when contemplating oral 1,25-$(OH)_2D_3$ therapy. While no hypercalcemia was observed in heart graft recipients, we did note elevated serum calcium levels and calcium deposits in the kidney tissue of some 1,25-$(OH)_2D_3$-treated animals. This points to a narrow therapeutic window but does suggest that if sufficient 1,25-$(OH)_2D_3$ can be administered without inducing hypercalcemia, then 1,25-$(OH)_2D_3$ may be an effective immunosuppressive agent. Van Etten *et al.* [36] have described a synergistic effect when 1,25-$(OH)_2D_3$ analogs were combined with CSA or mycophenolate mofetil (MMF) both *in vitro* and *in vivo*. Gregori *et al.* [8] have prolonged islet graft survival when 1,25$(OH)_2D_3$ was used in combination with MMF. This supports our observation that 1,25-$(OH)_2D_3$ may have efficacy as an immunomodulatory compound in transplantation. Other studies have identified a role in regulating immune responses in disease states characterized by autoimmunity as well as in transplant rejection [6]. In particular, 1,25-$(OH)_2D_3$ has been shown to inhibit the maturation of dendritic cells (DC) and to influence development of Th1 versus Th2 responses [9,10]. 1,25-$(OH)_2D_3$ also can induce the generation of regulatory T cells [8]. We and others have shown that 1,25-$(OH)_2D_3$ is effective in prolonging allograft survival in models of both acute rejection and CAN [7–9,28,37]. Redaelli *et al.* [37] have describe alterations in cytokine levels in 1,25$(OH)_2D_3$-treated recipients of renal allografts where prolongation from 6 to 10 days was achieved. We did not observe changes in serum levels of IFN-γ or IL-4 in our treated recipients (D.A. Hullett, unpublished observations). However we

did note attenuation of the early rise in serum creatinine that occurs in this model suggesting immunomodulatory effects of $1,25(OH)_2D_3$. Studies both *in vitro* and in other nonimmune fibrotic renal diseases clearly suggest the potential for $1,25(OH)_2D_3$ to regulate matrix remodeling. In this study, we have focused our analysis on the novel matrix modulating effects of $1,25(OH)_2D_3$. There is no doubt based on the observations described here that the beneficial effects of $1,25(OH)_2D_3$ in the setting of CAN represent a complex interaction between immunomodulation and regulation of matrix remodeling. We have noted here that $1,25(OH)_2D_3$ may have additional effects in addition to regulating the immune response especially in light of the observation that 500 ng/rat/day $1,25(OH)_2D_3$ was no more effective than low dose (1.5 mg/kg) in preventing the early acute rejection (Fig. 1) episode yet was the most effective in preventing CAN (Table 2). We realize that it is difficult to separate these effects in an *in vivo* model. Yet these grafts demonstrated a reduction in interstitial fibrosis and tubular atrophy compared with untreated and low dose CSA-treated allogeneic recipients and preservation of glomerular architecture.

The renal-specific effects we have described suggest that exogenous $1,25\text{-}(OH)_2D_3$ may have a direct role in regulating matrix deposition in CAN. The $1,25\text{-}(OH)_2D_3$ treated animals manifested a significant decrease in Smad 2 protein levels and an alteration in MMP expression. The demonstrated changes suggest a direct link between vitamin D, Smads and MMP regulation. Certainly, these data go a long way towards confirming the link between vitamin D and TGF-β1 signaling pathways that had been previously described *in vitro* [12,23,24]. The novel finding that $1,25\text{-}(OH)_2D_3$ also alters MMP levels is a logical extension of the potential role that $1,25\text{-}(OH)_2D_3$ may have in regulating intrarenal fibrosis.

Such a finding, combined with $1,25\text{-}(OH)_2D_3$'s demonstrated effects on immune cells, positions $1,25\text{-}(OH)_2D_3$ as a novel immunotherapy for CAN.

References

1. Jain S, Furness PN, Nicholson ML. The role of transforming growth factor beta in chronic renal allograft nephropathy. *Transplantation* 2000; **69**: 1759.
2. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor beta-1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997; **100**: 768.
3. Cohen AH, Nast CC. TGF-beta in renal allograft rejection. *Miner Electrolyte Metab* 1998; **24**: 197.
4. Suthanthiran M. Clinical application of molecular biology: a study of allograft rejection with polymerase chain reaction. *Am J Med Sci* 1997; **313**: 264.
5. Shihab FS. Molecular mechanisms of fibrosis in experimental chronic renal allograft injury. *Transplantation* 1998; **64**: 1829.
6. DeLuca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J* 2001; **15**: 2579.
7. Hullett DA, Cantorna MT, Redaelle C, Humpal-Winter J, Sollinger HW, DeLuca HF. Prolongation of allograft survival by 1,25-dihydroxyvitamin D_3. *Transplantation* 1998; **66**: 824.
8. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory t cells induced by 1alpha,25-dihydroxyvitamin d(3) and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol* 2001; **167**: 1945.
9. Gregori S, Giarratana N, Smiroldo S, Uskokovic M, Adorini L. A 1α,25-dihydroxyvitamin D3 analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice. *Diabetes* 2002; **51**: 1367.
10. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HFJ, O'Garra A. 1α,25-dihydroxyvitamin D3 has a direct effect on naive CD4+ T cells to enhance the development of Th2 cells. *J Immunol* 2001; **167**: 4974.
11. Strugnell SA, DeLuca HF. The vitamin D receptor–structure and transcriptional activation. *Proc Soc Exp Biol Med* 1997; **215**: 223.
12. Yanagisawa J, Yanagi Y, Masuhiro Y, *et al.* Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 1999; **283**: 1317.
13. Aschenbrenner JK, Sollinger HW, Becker BN, Hullett DA. 1,25-(OH(2))D(3) alters the transforming growth factor beta signaling pathway in renal tissue. *J Surg Res* 2001; **100**: 171.
14. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci U S A* 1996; **93**: 7861.
15. Diamond JR, Tilney NL, Frye J, *et al.* Progressive albuminuria and glomerulosclerosis in a rat model of chronic renal allograft rejection. *Transplantation* 1992; **54**: 710.
16. Paul LC. Chronic renal transplant loss. *Kidney Int* 1995; **47**: 1491.
17. Kreis HA, Ponticelli C. Causes of late renal allograft loss: chronic allograft dysfunction, death, and other factors. *Transplantation* 2001; **71**: SS5.
18. Tilney NL, Whitley WD, Diamon JR, Kupiec-Weglinski JW, Adams DH. Chronic rejection - an undefined conundrum. *Transplantation* 1991; **52**: 389.
19. Schnaper HW, Hayashida T, Hubchak SC, Poncelet AC. TGF-beta signal transduction and mesangial cell fibrogenesis. *Am J Physiol Renal Physiol* 2003; **284**: F243.
20. Stahl PJ, Felsen D. Transforming growth factor-β, basement membrane, and epithelial-mesenchymal transdifferentiation: implications for fibrosis in kidney disease. *Am J Pathol* 2001; **159**: 1187.

21. Koglin J, Glysing-Jensen T, Raisanen-Sokolowski ARME. Immune sources of transforming growth factor-beta1 reduce transplant arteriosclerosis: insight derived from a knockout mouse model. *Circ Res* 1998; **83**; 652.
22. Nicholson ML, Bicknell GR, Barker G, Doughman TM, Williams ST, Furness PN. Intragraft expression of transforming growth factor beta1 gene in isolated glomeruli from human renal transplants. *Br J Surg* 1999; **86**: 1144.
23. Yanagi Y, Suzawa M, Kawabata M, Miyazono K, Yanagisawa J, Kato S. Positive and negative modulation of vitamin D receptor function by transforming growth factor-beta signaling through Smad proteins. *J Biol Chem* 1999; **274**: 12971.
24. Subramaniam N, Leong GM, Cock TA, *et al.* Cross-talk between 1,25-dihydroxyvitamin D_3 and transforming growth factor-beta signalling requires binding of VDR and Smad3 proteins to their cognate DNA recognition elements. *J Biol Chem* 2001; **276**; 15741.
25. Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY. Smad 7 inhibits fibrotic effect of TGF-β on renal tubular epithelial cells by blocking Smad 2 activation. *J Am Soc Nephrol* 2002; **13**: 1464.
26. Johnson LL, Dyer R, Hupe DJ. Matrix metalloproteinases. *Curr Opin Chem Biol* 1998; **2**: 466.
27. Becker BN, Jacobson LM, Becker YT, *et al.* Renin-angiotensin system gene expression in post-transplant hypertension predicts allograft function. *Transplantation* 2000; **69**: 1485.
28. Piek E, Ju WJ, Heyer J, *et al.* Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 2001; **276**: 19945.
29. Darwish HM, DeLuca HF. Recent advances in the molecular biology of vitamin D action. *Prog Nucleic Acid Res Mol Biol* 1996; **53**: 321.
30. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol* 2002; **13**: 2600.
31. Abe H, Iehara N, Utsunomiya K, Kita T, Doi T. A vitamin D analog regulates mesangial cell smooth muscle phenotypes in a transforming growth factor-beta type II receptor-mediated manner. *J Biol Chem* 1999; **274**: 20874.
32. Schwarz U, Amann K, Orth SR, Simonaviciene A, Wessels S, Ritz E. Effect of 1,25 (OH)2 vitamin D3 on glomerulosclerosis in subtotally nephrectomized rats. *Kidney Int* 1998; **53**: 1696.
33. Kuhlmann A, Haas CS, Gross ML, *et al.* 1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat. *Am J Physiol Renal Physiol* 2004; **286**: F526.
34. Chen R, Huang C, Morinelli TA, Trojanowska M, Paul RV. Blockade of the effects of TGF-β1 on mesangial cells by overexpression of Smad7. *J Am Soc Nephrol* 2002; **13**: 887.
35. Bouillon R, Okumura K, Norman AW. Structure-function relationships in the vitamin D endocrine system. *Endocr Rev* 1995; **16**: 200.
36. van Etten E, Branisteanu DD, Verstuyf A, Waer M, Bouillon R, Mathieu C. Analogs of 1,25-dihydroxyvitamin D3 as dose-reducing agents for classical immunosuppressants. *Transplantation* 2000; **69**: 1932.
37. Redaelli CA, Wagner M, Gunter-Duwe D, *et al.* Schilling MK. 1alpha,25-dihydroxyvitamin D3 shows strong and additive immunomodulatory effects with cyclosporine A in rat renal allotransplants. *Kidney Int* 2000; **61**: 288.